https://doi.org/10.17221/10/2023-JFS

Pathogenicity of the entomopathogenic fungi against *Myllocerus fotedari* Ahmad, 1974 (Coleoptera: *Curculionidae*) under laboratory conditions in India

Shaziya Gull[1], Tariq Ahmad[1], Abdul Lateef Khanday[2], Pavittu Meethal Sureshan[3], Gowhar Rashid[2]*

[1]Section of Entomology, Department of Zoology, Faculty of Biological Sciences, University of Kashmir, Srinagar, India
[2]Department of Zoology, Government Degree College Kulgam, Jammu and Kashmir, India
[3]Zoological Survey of India, Western Ghat Regional Centre, Kozhikode, Calicut, Kerala, India

**Corresponding author: drtariqento@kashmiruniversity.ac.in*

Citation: Gull S., Ahmad T., Khanday A.L., Sureshan P.M., Rashid G. (2023): Pathogenicity of the entomopathogenic fungi against *Myllocerus fotedari* Ahmad, 1974 (Coleoptera: *Curculionidae*) under laboratory conditions in India. J. For. Sci., 69: 277–286.

Abstract: The weevil, *Myllocerus fotedari* Ahmad, 1974 (Coleoptera: *Curculionidae*) is widely recognised as one of the major walnut pests. Fungal pathogens have shown great potential for the management of some pest species. In the present study, the efficacy of three entomopathogenic fungi, namely *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae sensu lato* (Metchnikoff) Sorokin and *Lecanicillium lecanii* (Zimmerman) Zare and Gams were evaluated against the weevil, *M. fotedari,* under laboratory conditions. Each fungal suspension contained 1.0×10^9 spores of fungi in 1 mL. The experiment comprised of five treatments along with control (distilled water) and three replicates. An insecticide – chloropyrifos 20% emulsifiable concentrate (EC), was also used as positive control in the experiment. The experimental results revealed that the weevil, *M. fotedari,* showed mortality due to both virulence of entomopathogens and insecticide. The mortality caused by fungi varied from two days after treatment to eight days after treatment. However, the highest mortality of 100% was recorded for *B. bassiana*, followed by 85.1% for *M. anisopliae* after eight days of treatment. The efficacy of *L. lecani* was the lowest, leading to only 51.8% mortality, and was found significantly less virulent compared to the other two used entomopathogens. The present study is an attempt to use entomopathogens to control *M. fotedari* over conventional chemical insecticides.

Keywords: *Beauveria bassiana*; biological control; *Juglans regia; Metarhizium anisopliae*; *Lecanicillium lecanii*

The crops are usually infested with various pests and diseases making fruit crops and ornamental plants highly susceptible to pest attacks (Hill 1987; Oerke et al. 1994). Among different insect groups, coleopteran pests feed on the foliage and reduce the surface area of leaf that results in reduction of tree vigour, and thus host trees become prone to other diseases and insect attacks. *Myllocerus* spp. are economical pests feeding on numerous agricultural and horticultural crops with 4 144 genera, more than around 85 000 species with worldwide distribution (O'Brien, Wimber 1979). The occurrence of *Myllocerus* pests was first reported in Florida, feeding on numerous ornamental and fruit plants causing huge losses every year (O'Brien et al. 2006). In the Indian subcontinent, thirty-three species of this

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genus are recorded from diverse host plant species (Ramamurthy, Ghai 1988). Incidences of *Myllocerus* spp. are reported from various states of India with very scarce information on their bioecology and management (Ramamurthy, Ghai 1988; Azam 2007; Tara et al. 2010). These leaf pests are generally plant defoliators feeding on the foliage by nibbling irregular holes on the leaf surface, leaving behind midrib (Butani 1979). Their feeding activity lasts from April to November and remains indolent under the plant debris during winter (Atwal 1963). They are categorized as polyphagous pest because of their ability to feed on various host plants (Hill 1987). They can act as major pest when feeding on almond and walnut while as minor pest on apple trees (Butani 1979). The beetle has been documented to be a damaging foliage pest since adult *Myllocerus* spp. feed on leaf lamina, decreasing the quality and quantity of the fruit output by reducing the photosynthetic area of the leaf (Arévalo, Stansly 2009).

M. fotedari attacks walnut (*Juglans regia*), which is one of the main fruit crops of Jammu and Kashmir (India) and occupies almost 90% share of walnut industry in India. From an area of 61 723 ha, the state produces about 86 263 tons of walnut. *Myllocerus* sp. is a serious pest acting as an aerial and subterranean feeder which attacks each part of the plant, while its larvae are concealed feeders attacking roots inside the soil (Davidson 1966). Ahmad and Dar (1974) reported *M. fotedari* infesting apple, pear, plum and walnut. The adults are defoliators while larvae mainly infest the *Leguminacae* family. *Myllocerus* sp. attacking walnut trees cause blackening of leaf tips (Mir, Wani 2005; Gull et al. 2019). Many researchers have contributed to finding the lethal effects of the insecticides effective against *Myllocerus* sp. (Budhraja et al. 1984; Singh et al. 1991; Sinha, Marwaha 1995). Use of traditional chemical insecticides to control insect pests leads to an accumulation of persistent residues and as such an eco-friendly alternative is necessary. Introduction of biological control method as an essential part of integrated pest management (IPM) proved to be a more effective and rapidly developing alternative to chemical insecticide pest management. Thus, the primary focus is to find eco-friendly methods like the use of fungi to control various insect pests. The use of microbes in IPM strategies has been applied in both developing as well as developed countries and has been successfully used in South America and Asia (Fuxa 1984). Entomopathogens are highly eco-friendly with high persistence in the environment and mostly least harmless to non-target species. Thus, they can play an important role in controlling the population dynamics of pests. They are known to cause affliction in insects of nearly all orders, *viz. Diptera*, *Lepidoptera*, *Coleoptera*, *Hymenoptera*, *Hemiptera*, and *Orthoptera* (Ramanujam et al. 2014). These produce conidiospores which germinate on the cuticle breaching with the help of enzymes (like chitinase, proteinase and lipase) and mechanical pressure, developed hyphal tubes pierce integument, and hyphal bodies move towards haemolymph, where tremendous budding occurs to produce more spores leading to death of the host (Ferron 1978). Although various species are practically applied to control pests due to mycoinsecticidal activity, compared to other methods it is still in its infancy. In the present study, an attempt was made to know the bio-efficacy and comparative effects of some entomopathogenic fungi, *viz. Beauveria bassiana* (Balsamo), *Lecanicillium lecanii* (Zimmerman) and *Metarhizium anisopliae* (Metchnikoff) fungus against *M. fotedari* infesting walnut trees of Kashmir under laboratory conditions.

MATERIAL AND METHODS

Insect rearing. The specimens of the weevil, *M. fotedari,* were collected from the walnut trees of the Khimber (34.1898°N, 74.8609°E, elevation 1 682.80 m a.s.l.) in Srinagar, Jammu and Kashmir district. Thereafter, they were transferred to collection jars covered with muslin cloth to confirm air circulation, and were brought to Entomological Laboratory, University of Kashmir for evaluating the pathogenicity caused by various fungal treatments. Insect specimens were placed inside the rearing jars and were provided with fresh walnut leaves. GPS location of the sample site was taken from Google Earth Pro (7.3.2.5776, 2019).

Entomopathogenic fungi. Three commercial formulation of enthomopathogenic fungi with spore concentration 1.0×10^9 spores·mL^{-1}, namely *B. bassiana*, *L. lecanii*, and *M. anisopliae,* were obtained from Green Life Biotech Laboratory, Somanur, Coimbatore, India. For the evaluation of each entomopathogenic fungi treatment, three replicates were maintained. A total of 15 replicates were maintained including control comprising of distilled water and insecticidal treatment. The used insecticide was chloropyrifos 20% EC.

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Table 1. Treatment against *M. fotedari* using a Petri dish assay under laboratory conditions (29.85 °C ± 1.34 °C temperature and 77.57% ± 8.67% relative humidity)

Sample No.	Group*	No. of Petri dishes in each treatment/beetle	Preparation	Used quantity (mL per Petri dish)
1	G1	3/30	*Beauveria bassiana*	1.0
2	G2		*Lecanicillium lecanii*	
3	G3		*Metarhizium anisopliae*	
4	G4		insecticide	
5	G5		distilled water	

* Each group represents 3 Petri dishes with 10 *M. fotedari* adults subjected to 5 treatments

Bioassay. Each bio-preparation was diluted with distilled water forming 1 mL bio-preparation 1 000 mL^{-1} water to which a few drops of detergent were added as a wetting agent. 1 mL of each fungal suspension contained 1×10^9 spores·mL^{-1}. Each treatment was replicated three times. In each replicate 10 adult beetles were transferred using a camel hairbrush on walnut leaves into Petri dishes (11 cm diameter). In total, 30 beetles were used for each Petri dish assay (Table 1). Entomopathogenic fungal bioassay was conducted by using rapid jetting sprays two times, which was standardized at 1.0 mL for each replicate. For this purpose, a small calibrated hand spray with a one-litre capacity was used, which was fortified with a nozzle suited to low-volume spray applications on insect body as topical application method (Batta 2007; Khanday, Buhroo 2018; Khanday et al. 2018). All the treatments were sprayed with the same volumes (1 mL·$replicate^{-1}$) while for control, adult beetles were treated with distilled water. The mortality percentage data was recorded over a period of eight days and was evaluated 2, 4, 6, and 8 days after treatment. Fresh leaves were used each day for feeding the beetles. Mortality was assessed by observing beetles continuously for five minutes and if no movement occurred, they were treated as dead. Effected beetles developed mat-like mycelial growth on the body when kept under humid conditions (29.85 °C ± 1.34 °C and 77.57% ± 8.67%) for about one week due to germination of conidiospores which develop on superficial area cuticle and result in the development of long hyphal tubes penetrating directly into the insect's integument. Photographs were taken during the experiment by using a Leica DFC295 (Leica Microsystems, Gmbh, Germany) camera with an automontage software (4.10, 2017).

Statistical analyses. Experimental data was analysed by using Origin Pro software (Version 15, 2015). The collected data of mortality percentage and corrected mortality of *M. fotedari* adults in different treatments were analysed by means of the analysis of variance (ANOVA) at a 0.05% level of significance. Tukey's Honest Square Difference test was used to separate means of treatment. Abbott's formula (Abbott 1925) was used for mortality data correction with that in control [Equation (1)].

$$CM\ (\%) = \frac{T\ (\%) - C\ (\%)}{100 - C\ (\%)} \quad (1)$$

where:
CM (%) – corrected mortality;
T – mortality in treatment;
C – mortality in control.

RESULTS

In the present experiment, the results showing the mortality caused by fungal pathogens were recorded in treated replicates. The data shown in Table 2 indicate the corrected mortality of all the treatments used in the experiment, and it was observed (Table 2) that mortality in each treatment augmented with increasing time interval [Table S1 in the Electronic Supplementary Material (ESM)]. All the fungal treatments showed different mortality rates against *M. fotedari* in eight days after treatment. After two days of treatment at spore concentration 1×10^9 conidia·mL^{-1}, the mortality was 40 ± 10% for *B. bassiana*, 30 ± 10% for *M. anisopliae*, 10 ± 10% for *L. lecanii*, and 50 ± 10% for insecticide (positive control). Further, it was observed that insecticide showed a slightly higher mortality rate than other used treatments. On comparing the mean mortality percent of *B. bassiana, M. anisopliae, L. lecanii* and insecticide, it was recorded that *B. bassiana, M. anisopliae* and insecticide treat-

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Table 2. Effect of different treatments on the mortality rate of adults *Myllocerus fotedari* at different time interval

Treatment	Corrected mortality (%) (± SD)			
	2 DAT	4 DAT	6 DAT	8 DAT
Beauveria bassiana	40.0 (± 10)^{a}	58.5 (± 10.33)^{ad}	85.5 (± 17.14)^{ad}	100.0 (± 0.00)^{a}
Metarhizium anisopliae	30.0 (± 10)^{a}	44.8 (± 5.01)^{ac}	64.4 (± 11.73)^{ac}	85.1 (± 16.99)^{a}
Lecanicillium lecanii	10.0 (± 10)^{b}	24.0 (± 5.25)^{bc}	39.2 (± 5.58)^{bc}	51.8 (± 16.95)^{b}
Insecticide (chloropyrifos 20% EC)	50.0 (± 10)^{a}	78.8 (± 11.74)^{d}	100.0 (± 0.00)^{d}	100.0 (± 0.00)^{a}
Control (distilled water)	0.0 (± 0.00)^{b}	3.3 (± 0.57)^{e}	6.6 (± 0.57)^{e}	10.0 (± 0.00)^{c}

$^{a–e}$ mean values with different superscripts are significantly different ($P < 0.05$, Tukey's HSD); significant $P < 0.05$, non-significant $P > 0.05$; SD – standard deviation; DAT – day after treatment; EC – emulsifiable concentrate

ments were insignificant among themselves while *L. lecanii* showed a significant difference (one-way ANOVA; $P \leq 0.05$).

Four days after treatment, the maximum mortality was recorded for insecticide (78.8 ± 11.74%), followed by *B. bassiana* (58.5 ± 10.33%), *M. anisopliae* (44.8 ± 5.01%) and *L. lecanii* (24 ± 5.25%). One-way ANOVA revealed that treatment with *B. bassiana, M. anisopliae* and insecticide led to significantly higher mortality rates than other treatments ($P \leq 0.05$) while insignificant differences were observed between *B. bassiana* and insecticide. However, a significant difference ($P \leq 0.05$) was observed between insecticide and *M. anisopliae*, and also between *M. anisopliae* and *L. lecanii* (Figure 1).

After six days of treatment, an appreciable reduction in the number of beetles occurred, with a peak of 100% mortality in the case of insecticide, followed by *B. bassiana* (85.5 ± 17.14%), *M. anisopliae* (64.4 ± 11.73%), and *L. lecanii* (39.2 ± 5.58%). On analyzing the results with one-way ANOVA, an insignificant difference was found between insecticide and *B. bassiana*, while a significant difference was observed between insecticide and

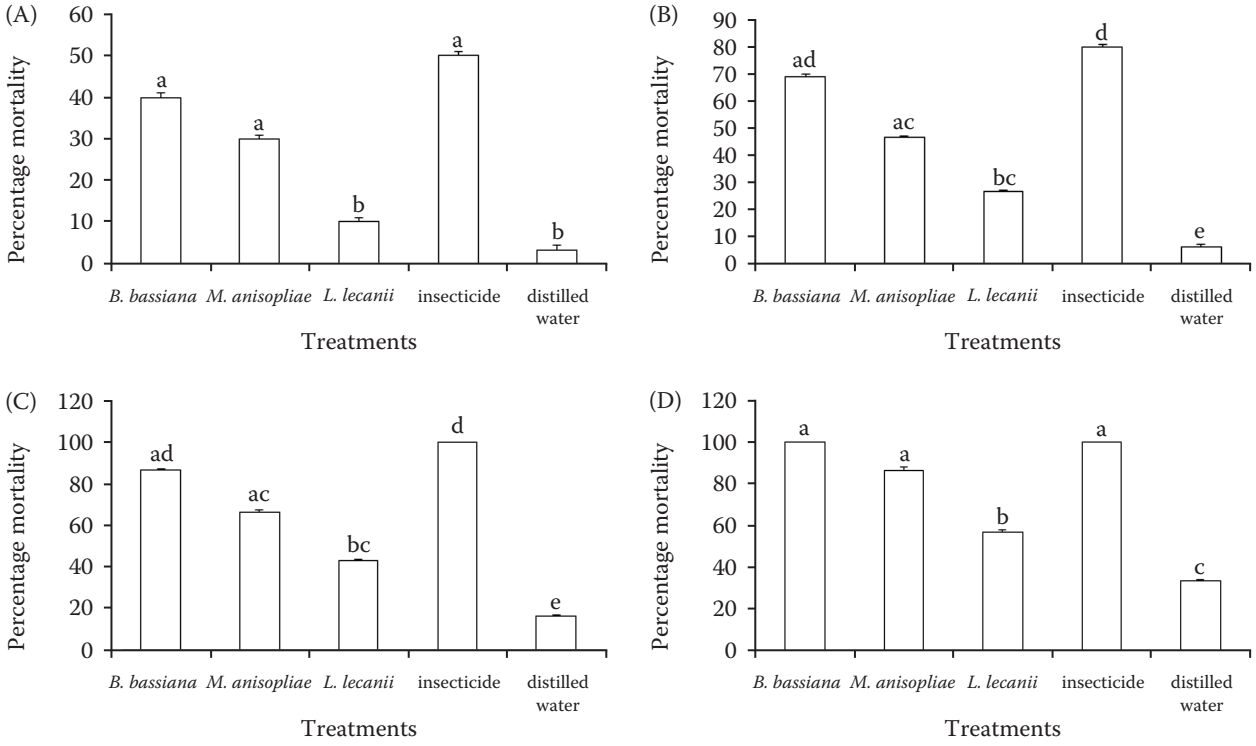

Figure 1. Mean percentage of corrected mortality of *Myllocerus fotedari* adults using Petri dish assays (A) 2 days after treatment; (B) 4 days after treatment; (C) 6 days after treatment; (D) 8 days after treatment

a–e – standard deviation is added on bars and the bars that do not share the same superscript are significantly different at $P < 0.05$ as indicated by Tukey's Honest Square Difference test

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other treatments ($P \leq 0.05$). Furthermore, *M. anisopliae* and *B. bassiana* were statistically the same at $P \leq 0.05$. Out of all the treated entomopathogenic fungi, *L. lecanii* showed significantly lower mortality rates in beetles (Figure 1).

Eight days after treatment, a marked increase in the mortality percent rate occurred, ranging from 51.8% to 100%. Among five treatments, 100% mortality occurred in the case of insecticide and *B. bassiana*, followed by *M. anisopliae* (85.1 ± 16.99%) and least by *L. lecanii* (51.8 ± 16.95%). On comparing the means with one-way ANOVA, *B. bassiana, M. anisopliae* and insecticide were insignificant to each other but significant to *L. lecanii* at $P \leq 0.05$. Among the three entomopathogens used, *B. bassiana* and *M. anisopliae* at spore concentration 1.0×10^9 resulted in the highest mortality rates, as compared to *L. lecanii* (Figure 1 and Table 2). The order of efficacy caused by five treatments in the present experiment can be ranked as: insecticide > *B. bassiana* > *M. anisopliae* > *L. lecanii* > control.

The regression equation for *B. bassiana* was calculated as $y = 10.35x + 19.25$, having a regression coefficient (R^2) value of 0.988 (Figure 2A).

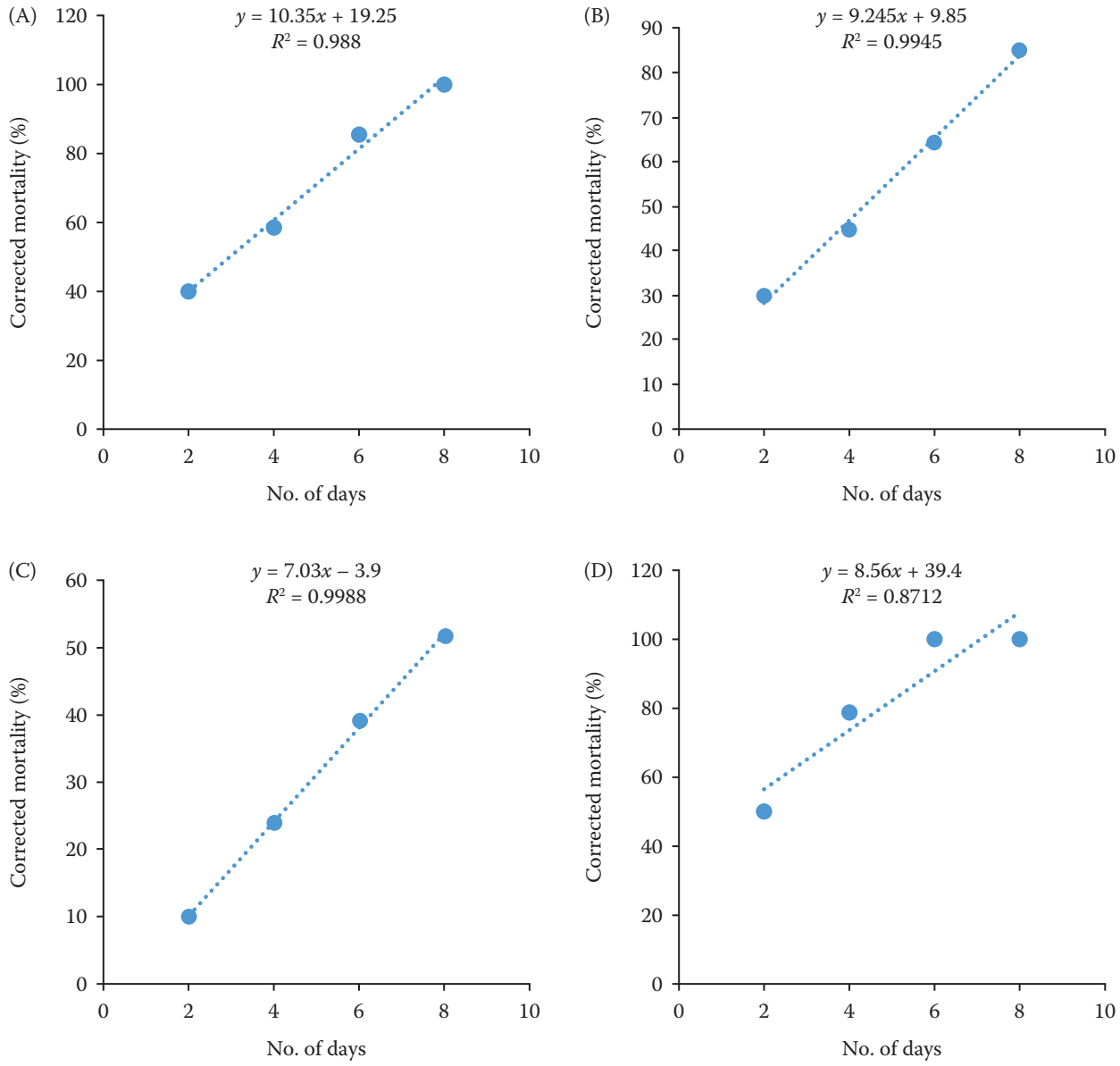

Figure 2. Graphs showing regression equation for mortality of *Myllocerus fotedari* by (A) *Beauveria bassiana*; (B) *Metarhizium anisopliae*; (C) *Lecanicillium lecanii*; (D) insecticide (chloropyrifos 20% EC)

EC – emulsifiable concentrate

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Positive correlation is exhibited between two variables x (No. of days) and y (mortality of beetles) as R^2 value tends to be less than 1. Regression equations for other treatments *viz. M. anisopliae* were $y = 9.245x + 9.85$ with R^2 value of 0.9945 (Figure 2B), *L. lecanii* had $y = 7.03x - 3.9$ along with R^2 of 0.9988 (Figure 2C) and for insecticide, the regression equation was $y = 8.56x + 39.4$, having R^2 value of 0.8712 (Figure 2D). Thus, it was concluded that an increasing number of days escalated the mortality rates of the beetles.

After 8 days of study, white mycelium mat growth was observed under a Leica M 205C stereo zoom trinocular microscope (Leica Microsystems, Gmbh, Germany) camera with an automontage software (4.10, 2017) on dead cadavers and fungal hyphae were observed protruding outside the body.

DISCUSSION

The efficacy of different entomopathogenic fungi was evaluated at 1.0×10^9 conidial spores·mL^{-1} against adults of *M. fotedari*, comprising of 5 treatments along with control and each treatment had three replicates. It was revealed from the results that there was a variation in pathogenicity of the entomopathogens used. *B. bassiana* (Figure 3C) and *M. anisopliae* (Figure 3A) showed high efficacy against *M. fotedari* in *in vitro* conditions leading to the highest mortality of 58.5% and 44.8%, respectively, after four days (96 h) of treatment, and 100% and 85.1% after eight days (192 h) of treatment, respectively. Further, dead cadavers developed white and green mat when treated with *B. bassiana* and *M. anisopliae*, respectively, due to hyphae penetration inside the cuticle consuming nutritional reserves of the body that leads to death of insects due to toxin production (Gabarty et al. 2014). Among the different known entomopathogens, *B. bassiana* and *M. anisopliae* gained attention for controlling weevils as they have a high potential to control long-lasting management, especially when used in combination with chemical insecticides (Mayer, Mannion 2011), and our results demonstrate that high mortality was observed on treating weevils with them. *B. bassiana* is well known for its insecticidal activities, having a high potential to kill a huge range of pests (Eken et al. 2006; Sabbour, E-Abd-El-Aziz 2007; Keyhani 2015), although, its efficacy varies on treating it with different Coleopteran species like in *Sitophilus zeamais* (Motschulsky 1855) (Coleoptera: *Curculionidae*) where 88% mortality occurred, when 10^4 conidial spores·mL^{-1} concentration was applied on 8th day of treatment (Adane et al. 1996). Conversely, 100% mortality was observed after 4.4 days on the larvae of *Saperda populnea* (Linnaeus 1758) (Coleoptera: *Cerambycidae*) under laboratory conditions (Eken et al. 2006). Similarly, our findings are in line with Yanar et al. (2015) who carried out an experiment on various isolates of *B. bassiana* and found 91.7% mortality rate on treating *Syrista parreyssii* (Spinola 1843) with them. Wu et al. (1995) also found 80% mortality caused in field trials, when *B. bassiana* was sprayed with spore concentration of 1.2×10^8 spores·mL^{-1} to control *Myllocerus* sp. Likewise, *B. bassiana* (Strain 871) was sprayed on tea crops with a spore suspension of 100–200 million spores·ha^{-1} leading to 95% mortality in brown weevil, *Mylloceus aurolineatus* (Voss 1959) after 10 days of treatment (Wu, Sun 1994).

It is also evident from our current findings that *B. bassiana* had more pathogenicity against the weevil *M. fotedari*, compared to other treated entomopathogens. Similar results were drawn by Mishra (1993) who found that among different entomopathogens, *B. bassiana* is the most promising and potential bio-control agent of *Hypsiphylla robusta* (Moore 1886). Our result also revealed that *B. bassiana* caused the highest mortality of 100% at 1.0×10^9 spores·mL^{-1} which is in line with the earlier findings of Logan et al. (1999) who reported 93.7% mortality at the same spore concentration in contrary to least mortality caused by *M. anisopliae* on the grey back cane grub, *Demolepida albohirtumn* (Waterhouse 1875) (Coleoptera: *Scarabidae*). Similarly, present results are in support of Senthilkumar and Murugesan (2010) who indicated that the highest concentration (1.0×10^9 spores·mL^{-1}) leads to the highest mortality in controlling the population of *Calopepla leayana* (Latreille 1807). The present inferences are in line with the findings of Sankaran et al. (1989) who carried out the management of *Myllocerus viridans* (Boheman 1840) with *B. bassiana* at spore concentration of 1.0×10^5 spores·mL^{-1}. Other workers have also studied pathogenicity caused due to *B. bassiana* on various curculionid beetles like banana weevil, *Cosmopolites sordidus* (Germar) (Godonou et al. 2000); pecan weevil, *Curcu-*

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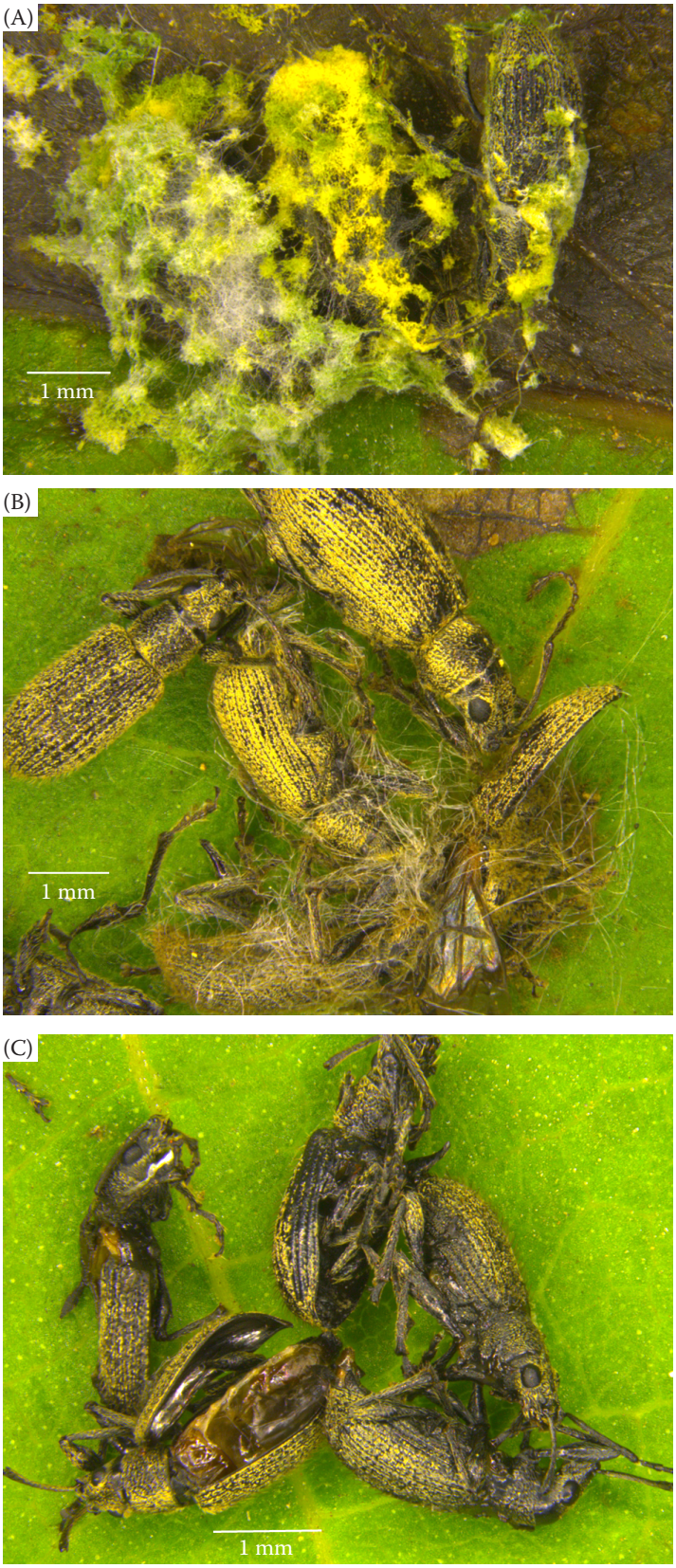

Figure 3. Entomopathogenic fungi *Metarhizium anisopliae, Lecanicillium lecanii*, and *Beauveria bassiana* infesting the weevil, *Myllocerus fotedari* infestation by (A) *Metarhizium anisopliae*; (B) *Lecanicillium lecanii*; (C) *Beauveria bassiana*

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lio caryae (Horn 1873) (Shapiro-Ilan et al. 2003) and pine weevil, *Hylobius abietis* (Linnaeus 1758) (Turchinskaya, Sherlyngina 1974).

In a Petri plate bioassay, 88–100% mortality occurred in *Ips typographus* (Linnaeus 1758) when treated with *B. bassiana*, which is in close proximity to our results (Vaupel, Zimmermann 1996; Kunca et al. 2009). Gopalakrishnan and Narayanan (1988) treated adults of *Mylloceus subfaciatus* (Guerin 1958) with *B. bassiana* where it was found that virulence in 5–8 days at a temperature of 25–27 °C and 80–90% relative humidity was 100%, which was in consonance with our findings where *B. bassiana* caused 100% mortality on the 8th day after application and is attributed to the fact that *B. bassiana* and *M. anisopliae* can cause virulence in insects at low ambient humidity as long as host cuticle contains ample moisture (Hallsworth, Mangan 1999). Further, the conclusion drawn by Batta (2007) was also the same, reporting high pathogenicity of *B. bassiana* against adults of almond bark beetle, *Scolytus amygdali* (Geoffroy 1792).

In the present study, a spore concentration of 1.0×10^9 spores·mL^{-1} was used to evaluate the mortality which was in line with various researchers who found that a higher concentration of spore suspension is likely to cause more virulence and infection of host (Ahmed, Elkatatny 2007; Steinwender et al. 2010). Further, *L. lecanii* (Figure 3) caused the lowest mortality compared to the other two entomopathogens, which is attributed to the fact that it is the potential biocontrol agent of aphids, thrips and whiteflies that is used mostly for the management of ornamental and salad crops (Chandler et al. 1993). Kreutz (2004) used entomopathogenic fungi, *B. bassiana,* and insecticide to control the insect population of *Ips typographus* (Linnaeus 1758) and achieved 100% and 93% mortality on treatment with insecticide and fungal isolate, respectively. Our results were similar and in covenant to their observations as within the first few days, the mortality caused by insecticide (positive control) was high but later mortality after 8 days of treatment reached 100% in both cases of insecticide and *B. bassiana*, and 85.1% with *M. anisopliae* and least with *L. lecanii* (51.8%). Similarly, the same conclusion is drawn by Kavallieratos et al. (2014) who carried out the work on *Sitophilus oryzae* (Linnaeus 1763) by controlling its population using *B. bassiana, Isaria furmosorea* and *M. anisopliae.* Observed mortality ranged from 0 to 100% and high efficacy, i.e. speed of killing, was shown by *B. bassiana* and *M. anisopliae*, which was in total agreement with our experimental findings. In the present investigation, it was further found that insecticide used in Petri plate assay caused higher mortality soon after the first day of treatment but widespread use of chemical pesticides caused a change in the ecosystem leading to death of non-target species (Aktar et al. 2009). Nevertheless, research has been carried out showing that entomopathogens can act as good biological control agents and are target specific with high virulence and various researchers are working to know the synergetic effect of entomopathogens with chemical insecticides (Rossetti et al. 2015).

CONCLUSION

Insects are highly susceptible to various fungal pathogens, having potent killing prowess against a wide range of hosts. This study discusses the use of various entomopathogenic fungi and a chemical insecticide on *M. fotedari* which caused significant mortality. Among the three entomopathogens, *B. bassiana* was found to be an apposite and more promising pesticide due to its high virulence. Thus, it can be used as a safe method to control insect pest instead of using chemical insecticides. However, field trials are indispensable to validate the virulence caused by these entomopathogens which led to high mortality in laboratory conditions, and further, field studies are a prerequisite to making them a major component of integrated pest management for *M. fotedari*.

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Received: January 25, 2023
Accepted: May 17, 2023
Published online: July 1, 2023